Nicotinic acid and nicotinamide belong to the water-soluble vitamins, and they have many physiological and pharmacological functions in various organisms. In this study, we investigated the differentiation-inducing ability of nicotinic acid-related compounds in chronic myelogenous leukemia K562 cell line. Proliferation of K562 leukemia cells was inhibited by several nicotinic acid-related compounds. Hemoglobin content was increased by nicotinic acid and by isonicotinic acid. Isonicotinic acid increased γ-globin mRNA expression as much as sodium butyrate did. The nuclei of nicotinic acid and of isonicotinic acid-treated cells decreased in size and the chromatin became more condensed. It was verified that nicotinic acid and isonicotinic acid induced erythroid differentiation in K562 cells. Expression of glycoporphin A was increased by sodium butyrate. In contrast, it was decreased by nicotinic acid and by isonicotinic acid, suggesting that these compounds differentiate K562 to erythrocytes through different pathways than sodium butyrate does. Our data perhaps provide useful information as to the mechanisms of cell differentiation.

Key words: nicotinic acid; isonicotinic acid; cell differentiation; K562

Nicotinic acid and nicotinamide are members of the vitamin B family. They are precursors of nicotinamide adenine dinucleotide (NAD). NAD is an important molecule involved in energy metabolism. Furthermore, it is used in important biochemical reactions, viz., ADP-ribosylation and NAD-dependent deacetylation of various proteins. In eukaryotic cells, poly(ADP-ribosyl)ation is related to DNA repair, cell death, transcription, DNA replication, cell cycle, and so on. Recently, it was reported that life span is regulated by sirtuin families, which are important factors in NAD-dependent deacetylation.21) We have been investigating nicotinic acid-related compounds to identify new physiological and pharmacological functions. In a previous study, we found that several nicotinic acid-related compounds, particularly picolinic acid, dipicolinic acid, and isonicotinamide induced apoptosis in HL-60 cells and other human cancer cells.3-5) Nicotinic acid hydrazide and isonicotinic acid hydrazide showed radical-scavenging activities.3) The HL-60 cell line has been used as a model in studying myeloid cell differentiation. Treatment with all-trans-retinoic acid (ATRA) and with dimethyl sulfoxide (DMSO) has been found to cause HL-60 to differentiate into granulocytes.3) In contrast, treatment with vitamin D3 induces differentiation into monocytes.8) We have found that three nicotinic acid-related compounds, isonicotinic acid, nicotinamide, and nicotinamide N-oxide induced granulocytic differentiation in HL-60 cells.9) K562 are human erythroleukemia cells derived from a patient with chronic myelogenous leukemia in blastic crisis.10) It has been extensively demonstrated that K562 cell line can be induced to differentiate towards megakaryocyte and erythroid lineages by various differentiation inducers. Treatment with phorbol-myristate-13-acetate (PMA) has been found to cause K562 to differentiate into megakaryocytic lineage.11) In contrast, treatment with hemin or sodium butyrate induces differentiation into erythroid lineage.12,13) K562 cell line provides a useful system for studying human erythroid differentiation because it expresses markers of erythroid lineage, such as hemoglobin. In this study, we investigated the differentiation-inducing ability of nicotinic acid-related compounds in K562 cell line.

Materials and Methods

Cell culture. K562 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). They were cultured in RPMI1640 medium (In vitrogen, Carlsbad, CA) containing heat-inactivated 5% fetal bovine serum, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and maintained at 37 °C in a humidified cell culture incubator containing 5% CO2. The cells were passaged at 2- to 3-d intervals to maintain exponential growth, and all experiments were performed with cells between passage 12 and passage 30. Cell viability was confirmed by flow cytometry using propidium iodide exclusion. It was always greater than 95%.

The cells were treated with 1–25 mM nicotinic acid-related compounds at a density of 1 × 105 cells/ml. Isocinchomeronic acid, isonicotinic acid, nicotinamide, nicotinic acid, nicotinamide N-oxide, picolinic acid, and quinolinic acid were purchased from Nacalai Tesque (Kyoto, Japan). Isonicotinic acid hydrazide, isonicotinic acid N-oxide, lutidinic acid, N'-methylnicotinamide, isonicotinic acid hydrazide, and picolinamide were from Tokyo Chemical Industries (Tokyo). Nicotinamide N-oxide and trigonelline were from Sigma Chemical (St. Louis, MO). Sodium butyrate was from Wako Pure Chemical Industries (Osaka, Japan).

MTT assay. Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.14) The cells were incubated with MTT solution (0.5 mM) for 4 h, 0.04 N HCl in isopropanol was added, and the amount of formazan formed was
assayed spectrophotometrically at 550 nm with a microplate reader for 96-well plates (Model MPRA4AiII, Tosoh, Tokyo).

Hemoglobin spectroscopy. The cells were pelleted from the culture medium by centrifugation, washed in phosphate-buffered saline (PBS) (–), (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, and 1.5 mM KH₂PO₄, pH 7.2). They were suspended and lysed in sterile distilled water at 0°C for 10 min. After pelleting of the debris for 10 min at 4°C (10,000 x g), the supernatant was collected, and absorption spectra from 200 to 600 nm were acquired using a UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan). The change in absorbance at 413 nm for each lysate was normalized to the protein concentration, yielding a specific measurement of hemoglobin content.

Real-time reverse transcription PCR. Total RNA was isolated with an Isogen RNA extraction kit (Nippon Gene, Tokyo). The concentration of RNA was measured using the optical density of samples at 260 nm. First-strand cDNA was synthesized using Moloney murine leukemia virus (M-MLV) reverse transcriptase. Briefly, 1 µg of total RNA was transcribed with a random hexamer primer (Takara Bio, Otsu, Japan), in a reaction mixture. PCR was done with 100 ng of first-strand cDNA. All PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in 96-well plates (Model MPRA4AiII, Tosoh, Tokyo). PCR amplification of the cDNAs was performed according to the manufacturer’s suggested protocol. The gene expression levels of γ-globin were assayed by quantitative real-time fluorescence-based PCR using ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR amplification of the cDNAs was carried out with primer pairs for γ-globin 5'-GCCATAAGCACCT-GGATGATC-3' and 5'-ATCCTGGAGCAGGCGACCTG-3'; and for GAPDH 5'-GCCCCCCGTTCGTCACACTG-3' and 5'-TTTCTCTTGCAGGGCCTTG-3'. Relative expression was normalized to the house-keeping gene GAPDH.

Flow cytometry. Expression of the erythroid as specific antigen Glycophorin A (CD235a) on K562 cell surface was determined by flow cytometry. The cells (1 x 10⁷) were washed with PBS (–) and incubated with anti-Glycophorin A antibody (1E4B-7-6, MBL, Aichi, Japan) in PBS (–) containing 1% bovine serum albumin for 30 min on ice. Then they were washed with PBS (–) and stained with the secondary antibody, FITC-conjugated goat anti-mouse IgG (MBL), in PBS (–) containing 1% bovine serum albumin for 30 min on ice. They analyzed on a FACScalibur (Becton Dickinson, San Jose, CA).

Morphologic studies. The cells were collected from the culture medium by centrifugation, and washed in PBS (–). They were fixed in a 3:1 mixture of ethyl alcohol and acetic acid. Then they were stained with Wright-Giemsa and examined under a microscope. Wright’s stain solution and Giemsa’s stain solution were purchased from Nacalai Tesque (Kyoto, Japan). Differentiated cells were identified on the basis of nuclear patterns.

Results

Effect of nicotinic acid related-compounds on cell proliferation and hemoglobin content

First, nicotinic acid-related compounds were examined for their anti-proliferative effects in K562 cells. As shown in Table 1, N⁴-methylnicotinamide, isonicotinic acid, isonicotinic acid hydrazide, lutfidin acid, nicotinamide, nicotinamide N-oxide, nicotinic acid, nicotinic acid hydrazide, picolinic acid, and picolinamide inhibited proliferation of K562 cells by 50–90%. The compounds that had no effect on cell proliferation were isocinchomeronic acid, isonicotinic acid N-oxide, nicotinic acid N-oxide, quinolinic acid, and trigonelline.

We investigated changes in the level of hemoglobin content in K562 cells treated with nicotinic acid-related compounds. After normalizing for total protein content, there was a 1.4-fold increase in the hemoglobin content of sodium butyrate-treated K562 cells, as reflected in the change in absorbance at 413 nm/mg protein content (ΔA₄₁₃/mg total protein). Nicotinic acid and isonicotinic acid increased hemoglobin content in the K562 cells. In contrast, nicotinamide, nicotinamide N-oxide, N⁴-methylnicotinamide, isonicotinic acid hydrazide, lutfidin acid, nicotinic acid hydrazide, and picolinamide decreased hemoglobin content in the K562 cells. Picolinic acid had no effect on hemoglobin content when cell proliferation was inhibited (Table 1).

Nicotinic acid and isonicotinic acid increased hemoglobin content of K562 cells when cell proliferation was inhibited. We performed more detailed analysis of antiproliferative effects and changes in the level of hemoglobin content of K562 cells treated with nicotinic acid and with isonicotinic acid. We found that three nicotinic acid-related compounds, nicotinamide, and nicotinamide N-oxide induced granulocytic differentiation in HL-60 cells. We examined these compounds for comparison. Nicotinic acid, isonicotinic acid, nicotinamide, and nicotinamide N-oxide inhibited proliferation of K562 cells. This phenomenon was linked to time-dependent inhibition of cell proliferation.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>Cell proliferation\textsuperscript{a} (% of control)</th>
<th>Hemoglobin\textsuperscript{a} (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>N⁴-Methylnicotinamide</td>
<td>15</td>
<td>64.2 ± 6.22</td>
<td>71.5 ± 9.49</td>
</tr>
<tr>
<td>Isonicotinic acid</td>
<td>10</td>
<td>81.2 ± 1.53</td>
<td>150 ± 5.86</td>
</tr>
<tr>
<td>Isonicotinic acid hydrazide</td>
<td>5</td>
<td>79.3 ± 3.10</td>
<td>71.6 ± 9.44</td>
</tr>
<tr>
<td>Lutfidin acid</td>
<td>10</td>
<td>86.2 ± 3.92</td>
<td>45.1 ± 2.01</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>10</td>
<td>84.0 ± 2.81</td>
<td>79.9 ± 1.41</td>
</tr>
<tr>
<td>Nicotinamide N-oxide</td>
<td>25</td>
<td>63.0 ± 2.61</td>
<td>66.8 ± 6.16</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>10</td>
<td>89.8 ± 8.50</td>
<td>121.5 ± 1.71</td>
</tr>
<tr>
<td>Nicotinamide hydrazide</td>
<td>5</td>
<td>78.0 ± 3.42</td>
<td>76.9 ± 8.46</td>
</tr>
<tr>
<td>Picolinic acid</td>
<td>1</td>
<td>82.3 ± 1.70</td>
<td>100.8 ± 10.10</td>
</tr>
<tr>
<td>Picolinamide</td>
<td>15</td>
<td>69.5 ± 1.45</td>
<td>74.2 ± 2.40</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>66.9 ± 2.40</td>
<td>140.3 ± 13.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cell proliferation was measured by MTT assay after 72 h treatment.

\textsuperscript{b}Hemoglobin content was determined by the change in absorbance at 413 nm after 120 h treatment. Results are presented as means ± SD for three independent experiments.
of K562 leukemia cells was inhibited dose-dependently by these four compounds (Fig. 1). The hemoglobin contents were increased by nicotinic acid and by isonicotinic acid treatment. On the other hand, nicotineamide and nicotineamide N-oxide decreased hemoglobin content in the K562 cells (Fig. 2).
Effect of nicotinic acid-related compounds on the expression level of γ-globin mRNA

Drug-induced differentiation of K562 cells was found to be associated with an increase in expression of γ-globin gene.15) We investigated the expression of γ-globin in K562 cells treated with 1 mM sodium butyrate, with 10 mM nicotinic acid, with 10 mM isonicotinic acid, and with 25 mM nicotinamide N-oxide. Treatment for 3 d with sodium butyrate and with isonicotinic acid enhanced γ-globin mRNA levels almost 3-fold (Fig. 3).

Effect of nicotinic acid-related compounds on the expression level of glycophorin A

Glycophorin A, also known as CD253a, is a sialoglycoprotein expressed on the surface of erythroblastic precursor cells of reticulocytes and mature red blood cells.16–18) We investigated the expression of glycophorin A in K562 cells treated with 1 mM sodium butyrate, with 10 mM nicotinamide, with 10 mM nicotinic acid, with 10 mM isonicotinic acid, and with 25 mM nicotinamide N-oxide. Glycophorin A expression was not observed under nicotinic acid, isonicotinic acid nicotinamide, or nicotinamide N-oxide treatment, in contrast to its activation by sodium butyrate (Fig. 4).

Effect of nicotinic acid-related compounds on morphological changes in K562 cells

We also investigated to determine whether nicotinic acid and isonicotinic acid would induce K562 toward erythroid differentiation by morphological stain with Wright-Giemsa at 5 d (Fig. 5A). The nuclei of nicotinic acid and of isonicotinic acid-treated cells decreased in size, and the chromatin became more condensed. K562 cells showed an increase in granularity as measured by sideward scatter and a decrease in size as measured by forward scatter in flow cytometer analyses (Fig. 5B).

Discussion

In this study, it was verified that nicotinic acid and isonicotinic acid induced erythroid differentiation in K562 cells. Nicotinic acid is a precursor of NAD which play essential metabolic roles in living cells. Nicotinic acid was found to reduce plasma cholesterol in the 1950s.19) It became the first drug available for lowering cholesterol-rich low density lipoproteins (LDL). Isonicotinic acid, an isomer of nicotinic acid, is a main metabolite of isonicotinic acid hydrazide, a major antituberculosis drug.20) It is clearly demonstrated that administration of isonicotinic acid to mice had no carcinogenic effect.21) Nevertheless, in isonicotinic acid-
treated mice, life expansion, decreased incidence of breast adenocarcinomas, and prolongation of latent periods were observed. We have found isonicotinic acid-induced differentiation in K562 cells. Sodium phenylacetate increased hemoglobin content and $\gamma$-globin expression, neither erythroid-specific marker nor CD71 expression were not observed under sodium phenylacetate treatment. These data suggest that nicotinic acid-related compounds and sodium butyrate differentiate K562 to erythrocytes through different pathways. Nicotinamide, nicotinamide $N$-oxide, and several compounds decreased hemoglobin content and $\gamma$-globin expression. K562 cells can be induced to differentiate to a wide variety of mature cells, i.e., PMA induces differentiation to megakaryocyte, and hemin induces erythroid differentiation, while 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induces differentiation to monocytes/macrophage, granulocytes, or megakaryocytes. Nicotinamide, nicotinamide $N$-oxide, and several compounds might induce differentiation to mature cells except for erythrocytes. It is necessary to elucidate the mechanism of differentiation induction in leukemia cells by nicotinic acid-related compounds.

References


Fig. 5. Effects of Nicotinic Acid-Related Compounds on Morphological Change in K562 Cells.
A. Morphologic change was assessed on slide preparations of cells stained with Wright-Giemsa. B. Changes in size and granularity of K562 cells. Sizes of the cells were measured by forward scatter (FSC, on the X-axis), granularity was measured by sideward scatter (SSC, on the Y-axis). a, Untreated K562 cells; b, 1 mM sodium butyrate; c, 10 mM nicotinic acid; and d, 10 mM isonicotinic acid.


